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In vitro development of preimplantation porcine embryos using alginate hydrogels as a three-dimensional extracellular matrix

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Abstract. Between Days 10 and 12 of gestation, porcine embryos undergo a dramatic morphological change, known as elongation, with a corresponding increase in oestrogen production that triggers maternal recognition of pregnancy. Elongation deficiencies contribute to embryonic loss, but exact mechanisms of elongation are poorly understood due to the lack of an effective *in vitro* culture system. Our objective was to use alginate hydrogels as three-dimensional scaffolds that can mechanically support the *in vitro* development of preimplantation porcine embryos. White cross-bred gilts were bred at oestrus (Day 0) to Duroc boars and embryos were recovered on Days 9, 10 or 11 of gestation. Spherical embryos were randomly assigned to be encapsulated within double-layered 0.7% alginate beads or remain as non-encapsulated controls (ENC and CONT treatment groups, respectively) and were cultured for 96 h. Every 24 h, half the medium was replaced with fresh medium and an image of each embryo was recorded. At the termination of culture, embryo images were used to assess morphological changes and cell survival. 17β-Oestradiol levels were measured in the removed media by radioimmunoassay. Real-time polymerase chain reaction was used to analyse steroidogenic transcript expression at 96 h in ENC and CONT embryos, as well as *in vivo*-developed control embryos (i.e. spherical, ovoid and tubular). Although no differences in cell survival were observed, 32% ($P < 0.001$) of the surviving ENC embryos underwent morphological changes characterised by tubal formation with subsequent flattening, whereas none of the CONT embryos exhibited morphological changes. Expression of steroidogenic transcripts *STAR*, *CYP11A1* and *CYP19A1* was greater ($P < 0.07$) in ENC embryos with morphological changes (ENC+) compared with CONT embryos and ENC embryos with no morphological changes (ENC−), and was more similar to expression of later-stage *in vivo*-developed controls. Furthermore, a time-dependent increase ($P < 0.001$) in 17β-oestradiol was observed in culture media from ENC+ compared with ENC− and CONT embryos. These results illustrate that preimplantation pig embryos encapsulated in alginate hydrogels can undergo morphological changes with increased expression of steroidogenic transcripts and oestrogen production, consistent with *in vivo*-developed embryos. This alginate culture system can serve as a tool for evaluating specific mechanisms of embryo elongation that could be targeted to improve pregnancy outcomes.

Additional keywords: elongation, pig, steroidogenesis.

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Introduction

In the pig, the preimplantation period of pregnancy is characterised by several developmental hallmarks. Between Days 10 and 12 of gestation, the porcine embryo undergoes a process known as elongation, transforming from an approximately 1-mm spherical structure to a long, thin filament that is >100 mm in length (Geisert *et al.* 1982*a*; Pusateri *et al.* 1990). Unlike elongation in other domestic species, rapid elongation of the pig embryo occurs via cellular remodelling and differentiation of the trophectoderm rather than cellular hyperplasia

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(Geisert *et al.* 1982*a*; Pusateri *et al.* 1990). During elongation, the embryo produces and secretes oestrogen, which serves as a key molecular signal for maternal recognition of pregnancy and modulates the production of proteins and growth factors within the uterus (Geisert *et al.* 1982*b*). Adequate elongation and successful implantation of the porcine embryo subsequently affect embryo spacing in the uterus, placental development and fetal growth, which have implications on uterine capacity, litter size and postnatal piglet health (Bennett and Leymaster 1989; Geisert and Schmitt 2002).

Approximately 20% of embryonic loss occurs during the preimplantation period of pregnancy in the pig, with a significant proportion of these losses occurring due to deficiencies in embryo elongation (Pope 1994). Early embryonic loss is poorly understood because exact physiological mechanisms by which the embryo elongates, and how these mechanisms are altered during embryonic loss, are not clear. Developing details of the mechanisms that regulate embryo elongation can allow for identification of specific biophysical, biomechanical and molecular factors that could serve as targets for improving pregnancy outcomes and neonatal piglet survival. An effective *in vitro* culture system that supports pig embryo elongation could be used to evaluate specific mechanisms of elongation. To date, attempts to initiate elongation in porcine embryos *in vitro* have been unsuccessful compared with other livestock species (Vejlsted *et al.* 2006). In cattle, limited elongation of embryos *in vitro* via physical induction was demonstrated using an agarose gel tube system (Brandão *et al.* 2004; Vajta *et al.* 2004; Machado *et al.* 2012). However, embryos cultured in this system had deficiencies in the development of the embryonic disk (Brandão *et al.* 2004; Vejlsted *et al.* 2006), which may have been the result of forced growth of the embryos to fill the tubes. Nevertheless, attempts to repeat these results using porcine embryos have been unsuccessful (Vejlsted *et al.* 2006).

Alginate is a commonly used biomaterial that is often used as an artificial extracellular matrix in tissue engineering applications (Lee and Mooney 2001). Alginate is a linear polysaccharide derived from brown algae composed of repeating units of β-D-mannuronic acid and α-L-guluronic acid (Amsden and Turner 1999). One of the favourable properties of alginate as a biomaterial is its ability to form a hydrogel by ionic cross-linking of the guluronic residues in the presence of a divalent cation (Gombotz and Wee 1998). The alginate gel forms a mesh-like structure that permits diffusion of nutrients and hormones essential for cell and tissue growth and development (Amsden and Turner 1999). Furthermore, alginate promotes negligible non-specific protein adsorption and cell adhesion, making it a suitable material for solely examining mechanical influence on tissue morphogenesis (Rowley *et al.* 1999; Miyajima *et al.* 2011). We hypothesise that previous failure of porcine embryos to elongate *in vitro* is due, at least in part, to inadequate culture systems lacking a three-dimensional (3D) structure for proper biomechanical support of embryo elongation. Traditional two-dimensional (2D) cultures result in a disruption of cell-to-cell interactions as cells attach to the substrate and migrate away from the tissue (Smitz and Cortvrindt 2002; Kreeger *et al.* 2006). Alternatively, 3D culture systems can maintain embryo architecture and allow for direct physical interaction with the surrounding environment, which better mimics *in vivo* development. Therefore, our objective was to use alginate hydrogels to establish an *in vitro* culture system based on tissue engineering principles (i.e. 3D scaffolds) that can mechanically support the culture of preimplantation porcine embryos, ensure proper internal biomechanics and initiate morphological changes. In the present study, *in vitro* development was evaluated by characterising cellular survival, morphological changes, gene expression of steroidogenic and immunological transcripts, and 17β-oestradiol (E2) production from porcine embryos encapsulated (ENC) in alginate hydrogels or remaining as non-encapsulated controls (CONT).

Materials and methods

Production and collection of embryos

All animal protocols were approved by the US Meat Animal Research Center (USMARC) Animal Care and Use Committee and met the US Department of Agriculture (USDA) guidelines (USDA 1995) for the care and use of animals. Fifteen normally cycling White cross-bred gilts consisting of Landrace, Yorkshire and Duroc genetics were checked daily for oestrus. Following the first detection of oestrus (designated as Day 0), gilts were artificially inseminated with semen from a single Duroc sire with three sires represented across the replicate collections (International Boar Semen, Eldora, IA, USA; mention of trade names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the same by USDA implies no approval of the product to the exclusion of others that may also be suitable) and again 24 h later with the same sire. In three independent replicate collections, five gilts were killed at the USMARC abattoir on Day 9, 10 or 11 of gestation. After the gilts had been harvested, their reproductive tracts were immediately removed and each uterine horn was flushed with 20 mL (~37°C) of HEPES-buffered RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 1× antibiotics and antimycotics (Sigma-Aldrich, St Louis, MO, USA). Embryos were recovered and classified according to size and morphology using a standard stereomicroscope. Embryos were then washed with HEPES-buffered RPMI-1640 medium containing 1× antibiotics and antimycotics and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen). From homogeneous litters, spherical embryos (~1 mm diameter; range 0.5–1.5 mm) recovered from gilts on Day 9 of gestation ($n = 3$ gilts per replicate) were randomly assigned to the *in vitro* culture treatments (ENC or CONT); a subset of these embryos was collected and snap-frozen individually in liquid nitrogen to serve as initial *in vivo*-produced spherical control embryos for transcript expression. From homogeneous litters, ovoid (5–10 mm diameter) or tubular (>10 mm diameter; range 11–20 mm) embryos were recovered from the remaining gilts on Day 10 or 11 of gestation, respectively ($n = 1$ gilt per day per replicate) and snap-frozen individually in liquid nitrogen to serve as later-stage *in vivo*-developed control embryos for transcript expression. Uterine flushings from all pregnant gilts were collected, centrifuged at 2100*g* for 20 min at room temperature to remove cellular debris and stored at −20°C for later E2 and protein analysis.

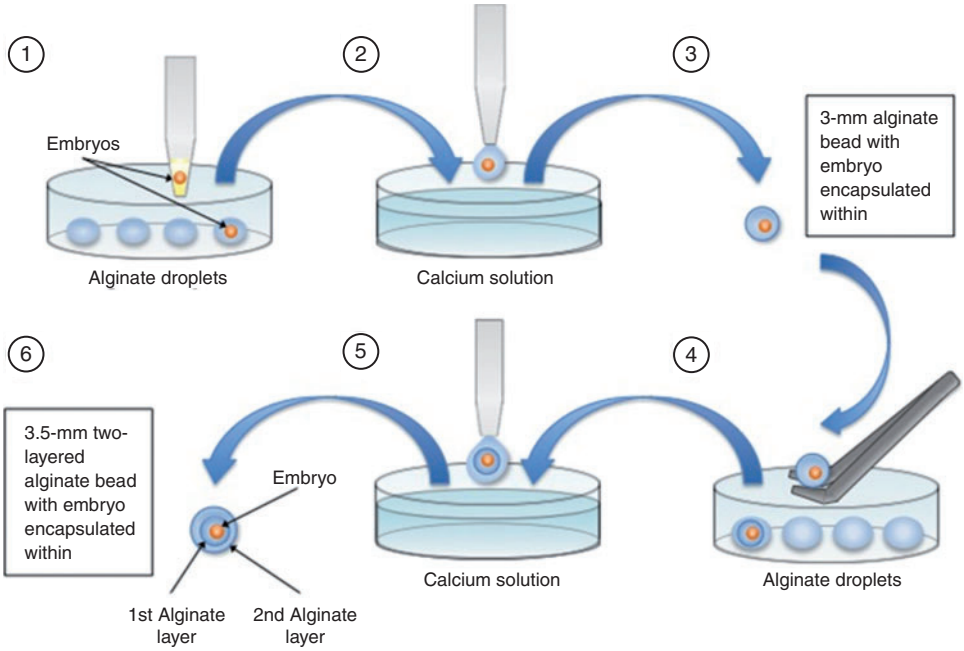

Fig. 1. Schematic illustration of the double-encapsulation method. ① The embryo is transferred into the alginate droplet; ② the droplet is pipetted into calcium solution; ③ an alginate bead forms around the embryo; ④ the alginate bead is transferred to a larger droplet of alginate; ⑤ the droplet with bead is pipetted into calcium solution; and ⑥ the second layer of alginate forms around the embryo. Following encapsulation, all embryos are washed and cultured individually in a single well within four-well NUNC plates (Thermo-Scientific, Rochester, NY, USA).

Embryo encapsulation and culture

Embryos assigned to the ENC group were encapsulated within alginate hydrogels using a double-encapsulation method (Fig. 1). Briefly, sodium alginate (Pronova UP MVG (>60% guluronic acid; 200 000–300 000 g mol^{-1}); NovaMatrix, Sandvika, Norway) was dissolved in sterile MilliQ water to a final concentration of 0.7% (w/v). Droplets of warmed, sterile alginate solution were placed onto polystyrene culture dishes. A single embryo was pipetted into each droplet of alginate in a minimal amount of medium as not to dilute the alginate solution. Each droplet was then pipetted into a warmed, sterile cross-linking solution (50 mM $CaCl_2$, 140 mM NaCl) and allowed to gel for 3 min. The resulting alginate beads with encapsulated embryos were then rinsed twice in HEPES-buffered RPMI-1640 medium containing 1× antibiotics and antimycotics and 10% FBS. Following rinsing, each bead was encapsulated in a second layer of alginate, using identical methods as described above for the initial encapsulation, to ensure complete encapsulation of each embryo. The final resulting beads were then washed once with 5% CO_2-gassed culture medium containing RPMI-1640, 1× antibiotics and antimycotics and 10% FBS. The ENC and CONT embryos were cultured in individual wells in four-well NUNC plates (Thermo-Scientific, Rochester, NY, USA) with 1 mL culture medium for 96 h at 38.5°C and 5% CO_2 in air with 100% humidity.

Characterisation of embryo viability and morphology

Every 24 h, a photographic image was recorded of each embryo using an SMZ1500 stereomicroscope (Nikon Instruments, Melville, NY, USA). Based on visual appraisal of these images, embryos were classified into one of three *in vitro* treatment groups: CONT (non-encapsulated control embryos), ENC– (encapsulated embryos with no observable morphological change) or ENC+ (encapsulated embryos with observable morphological changes). Embryos undergoing morphological changes were characterised by a tubular formation of the embryo within the gel and subsequent flattening of this tube, with many of these embryos migrating out of the gel forming a secondary spherical structure once freely in the culture medium (Fig. 2*c*). At the termination of culture, embryo survival was determined by assessing blastocyst fragmentation. Surviving embryos were characterised with no apparent sign of cellular degeneration, whereas dying embryos had significant cellular degeneration resulting in blastocyst compaction and darkening of the embryo. Embryo survival and death were confirmed in a subset ($n = 10$) of embryos using a fluorescein–ethidium bromide live–dead staining protocol, as reported previously (Edwards and Hansen 1996). Live or dead staining (green or red, respectively) was examined and imaged using an Axioplan2 fluorescence microscope (Zeiss, New York, NY, USA).

Transcript expression analysis

At the termination of the 96-h culture period, all embryos were imaged before being snap-frozen in liquid nitrogen. The CONT embryos were frozen in a small volume of culture medium (~5 μL), whereas ENC embryos were frozen within the alginate hydrogels. Preliminary analysis of RNA quality following extraction demonstrated no difference when embryos remained

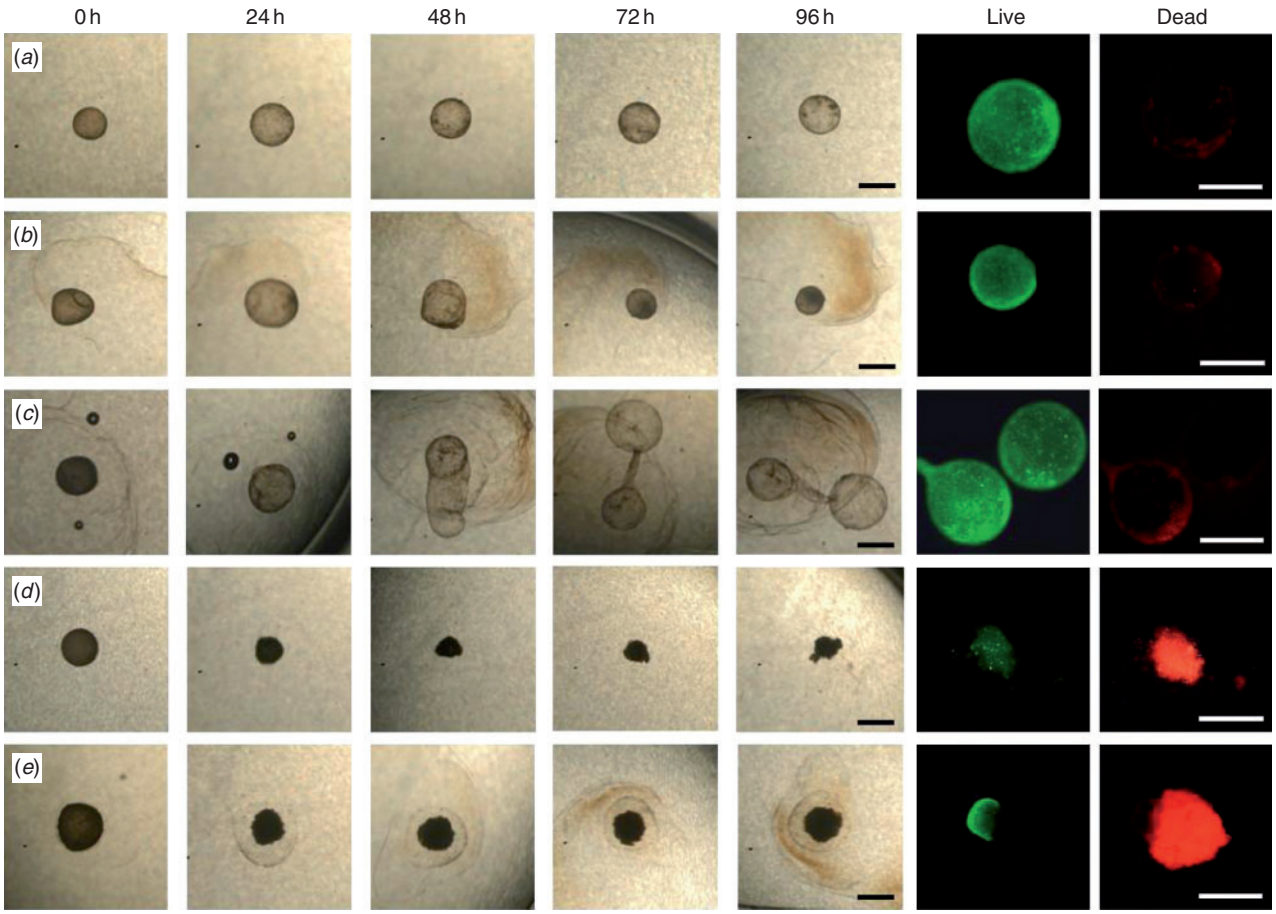

Fig. 2. Representative illustrations of morphological changes observed over 96 h of culture in embryos double-encapsulated in 0.7% alginate hydrogels or remaining as non-encapsulated control embryos, with corresponding cellular survival and death after 96 h culture. Surviving embryos that remained intact with no signs of cellular degeneration were classified into one of three *in vitro* treatment groups: (*a*) non-encapsulated control embryo with no observable morphological change (CONT); (*b*) encapsulated embryo with no observable morphological change (ENC−); or (*c*) encapsulated embryo with observable morphological changes (ENC+). Also shown are images of (*d*) CONT and (*e*) ENC− embryos that underwent significant cellular death, characterised by significant cellular degeneration resulting in blastocyst compaction and cellular darkening. The same representative embryo from each treatment group is shown across the different time points of culture and the live–dead staining. Scale bars = 1 mm. Note the morphological changes of the ENC+ embryo characterised by tubular formation of the embryo at 48 h that flattens by 72 h, resulting in the embryo migrating out of the hydrogel by 96 h. There were no observable differences in cellular survival (green stain) or death (red stain) between surviving embryos in the three treatment groups (*a*–*c*); however, a greater proportion of dead cells (red stain) was observed in embryos classified as undergoing cellular death (*d*, *e*).

encapsulated in the hydrogel versus de-encapsulating the embryos before collection and extraction (data not shown). Whole-cell (wc) RNA was extracted from individual embryos that survived (i.e. remaining intact with no cellular degeneration observed) throughout the duration of culture using the RNeasy Microkit (Qiagen, Valencia, CA, USA) with on-column DNase I treatment as described by the manufacturer. At the same time, wcRNA was extracted from *in vivo*-developed control embryos (i.e. spherical, ovoid and tubular) that had previously been collected. Quantification of wcRNA was performed with RiboGreen (Molecular Probes, Eugene, OR, USA) as per the manufacturer's instructions.

Using porcine-specific primers previously validated to amplify mRNA specific for the long-form variant of steroidogenic acute regulatory protein (*STAR*), cytochrome P450 side chain cleavage (*CYP11A1*), aromatase (*CYP19A1*) and interleukin-1β (*IL1B*; Blomberg and Zuelke 2005; Miles *et al.* 2008), transcript expression levels were assessed using a two-step, real-time quantitative polymerase chain reaction (qPCR) method with a Chromo4 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Briefly, reverse transcription was performed with 1 ng wcRNA from the *in vivo*-developed control (i.e. spherical, $n = 7$; ovoid, $n = 11$; tubular, $n = 6$), CONT ($n = 7$), ENC− ($n = 13$), and ENC+ ($n = 9$) embryos using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. In addition, a pooled sample of wcRNA consisting of 1 ng wcRNA from all embryo treatment groups represented was reversed transcribed

to generate a pooled cDNA sample that was used to generate a relative standard curve of cDNA for determining interassay CV and the relative quantity (RQ) of transcript expression. Each qPCR was assayed in duplicate and consisted of 50-pg equivalents of cDNA, 0.25 μM of the appropriate forward and reverse primers and 12.5 μL of 1× iTaq SYBR Green Supermix with ROX (Bio-Rad) in a 25 μL reaction. All PCR conditions included denaturation (95°C for 2 min) followed by amplification (95°C for 15 s, 60°C for 15 s and 70°C for 45 s) for 40 cycles. Melting curve analysis and gel electrophoresis were used to confirm amplification of a single product of the predicted size.

Expression levels for each transcript were based on the threshold cycle (C_T) values determined using Opticon Monitor 3 software (Bio-Rad). For each transcript, two assays were performed containing equal representation of all embryo treatment groups and a standard serial dilution of pooled cDNA consisting of 200-, 50-, 12.5-, 3.12- and 0.78-pg equivalents of cDNA. After converting the exponential C_T to the linear C_T using the formula 2^{-C_T} (Livak and Schmittgen 2001), the interassay CV for each transcript was determined using the pooled cDNA samples from each assay and the intraassay CV for each transcript was an average of all samples from both assays. The inter- and intraassay CV were 19.3% and 18.8%, respectively, for *STAR*, 16.1% and 18.0%, respectively, for *CYP11A1*, 14.9% and 20.0%, respectively, for *CYP19A1* and 7.3% and 20.4%, respectively, for *IL1B*. Calculations of RQ values were made using a relative standard curve method by plotting treatment C_T values against the logarithmic values of standard amounts of pooled cDNA (Čikoš *et al.* 2007). The RQ values are expressed as arbitrary units.

Assays for E2 and protein

Every 24 h, half the culture medium (500 μL) in each well was collected, stored at −20°C for hormone and protein analysis and replaced in culture with fresh pregassed culture medium (500 μL). In addition, wells containing culture medium alone (MEDIA) were included in each replicate and processed similarly to wells containing embryos. Analyses of E2 and protein content in the culture medium were performed in samples obtained after 24, 48, 72 and 96 h from the MEDIA group ($n = 8$) and cultures containing embryos represented in the qPCR analysis (i.e. CONT, $n = 7$; ENC−, $n = 13$; ENC+, $n = 9$). In addition, independent E2 and protein analyses were performed on uterine flushings collected from pregnant gilts on Days 9 ($n = 9$), 10 ($n = 3$) and 11 ($n = 3$). Culture media and uterine flushings were measured for E2 using a radioimmunoassay procedure described previously and validated in swine (Redmer and Day 1981; Miles *et al.* 2008). Culture media were ether extracted and measured for E2 in duplicate in one assay with an intraassay CV of 12.1%. Uterine flushings were ether extracted and measured for E2 in duplicate in one assay with an intraassay CV of 13.0%. In addition, culture media and uterine flushings were measured for total protein content using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). The protein content in the culture media was measured in duplicate in six assays with inter- and intraassay CVs of 9.3% and 7.7%, respectively. Protein content from uterine flushings was measured in duplicate in one assay with an intraassay CV of 2.4%.

Statistical analysis

Proportional data for embryo survival and morphological changes following culture were analysed using the Chi-squared test and are reported as the least-squares mean (LSM) ± s.e.m. after calculation with GLIMMIX model procedures (Steel *et al.* 1997; SAS 2003). Transcript expression data and E2 and protein levels in the culture media and uterine flushings were analysed using MIXED model procedures and are reported as the LSM ± s.e.m. (Steel *et al.* 1997; SAS 2003). When a significant *F*-statistic was generated, mean values were separated using Dunnett's multiple comparison test (Steel *et al.* 1997; SAS 2003). Mean values were considered significantly different at $P \leq 0.05$, with tendencies defined as *P*-values between 0.06 and 0.10. Transcript expression data were categorised into six treatment groups corresponding to *in vivo*-developed spherical, ovoid and tubular embryos and *in vitro*-cultured CONT, ENC− and ENC+ embryos, and the model included the fixed effects of treatment and replicate and the random effect of gilt within treatment. Culture E2 and protein data were categorised into four treatment groups (i.e. MEDIA, CONT, ENC− and ENC+) and the model included the fixed effects of treatment, culture time, replicate and treatment × culture time interaction, and the random effect of gilt within treatment × culture time interaction. Uterine flushing E2 and protein data were analysed with the model including day of gestation, replicate and the random effect of sire within day of gestation. To remove skewness and normally distribute the data, all transcript expression and E2 data were log transformed before statistical analysis and then back transformed to report observed means.

Results

Evaluation of embryo viability and morphology

Representative morphological changes observed over 96 h of culture of CONT and ENC embryos and their corresponding cellular survival and death, as determined by live–dead staining after 96 h culture, are shown in Fig. 2. Assessment of blastocyst degeneration at the termination of culture indicated that there was no significant difference ($P = 0.22$) in survival between the CONT and ENC embryos (Table 1). Embryo survival and death were further confirmed by live–dead staining a subset of embryos, which demonstrated no significant differences in cellular survival or death between treatment groups. Embryos classified as undergoing cellular degeneration had a greater proportion of dead cells (red), whereas surviving embryos had a greater proportion of live cells (green; Fig. 2). Morphological changes were only observed in the ENC embryos (Fig. 2*c*), whereas all CONT embryos remained spherical throughout the culture period (Fig. 2*a*; Table 1). The percentage of embryos that underwent morphological changes differed significantly between CONT and ENC groups when analysing all cultured embryos ($P < 0.05$) and only those embryos surviving ($P < 0.001$; Table 1). The morphological changes observed in the ENC embryos were characterised by a tubular formation of the embryo within the gel and subsequent flattening of this tube, with many of these embryos migrating out of the gel forming a secondary spherical structure once freely in the culture medium (Fig. 2*c*). Although 32% of the surviving ENC embryos

underwent morphological changes, a significant proportion of ENC embryos remained spherical throughout culture (Fig. 2*b*). As a result, encapsulated embryos were further classified as encapsulated embryos with no morphological changes (ENC−) or encapsulated embryos with morphological changes (ENC+).

Table 1. Summary of embryo survival and morphological changes observed following 96 h culture of either non-encapsulated control porcine embryos (CONT) or porcine embryos double encapsulated (ENC) in 0.7% alginate hydrogels

Values are reported as the least-squares mean ± s.e.m., as determined using GLIMMIX analysis for the main effect of treatment (i.e. control vs encapsulated embryos). Significance for the effect of treatment was determined using Chi-squared analysis

	CONT	ENC	*P*-value
No. embryos	24	71	
Embryo survival[A] (%)	33.3 ± 9.6 (8)	47.8 ± 5.9 (34)	0.22
No morphological change from all embryos (%)	100	83.1 ± 4.4	0.03
Morphological change from all embryos (%)	0	16.9 ± 4.4	0.03
Morphological change from surviving embryos (%)	0	32.3 ± 8.0	<0.001

[A]Embryo survival was determined as a lack of blastocyst degeneration. Numbers in parentheses indicate the number of surviving embryos in each treatment group.

Transcript expression levels

The expression of *STAR* mRNA was increased ($P < 0.05$) in all *in vitro* treatment groups (CONT, ENC− and ENC+) compared with the initial *in vivo* spherical control (Fig. 3*a*). However, *STAR* expression was greater ($P < 0.05$) in ENC+ compared with ENC− embryos and tended to be greater ($P = 0.07$) in ENC+ versus CONT embryos (Fig. 3*a*). As a result, the expression pattern of *STAR* in CONT and ENC− embryos was more similar to that in *in vivo* ovoid embryos, whereas ENC+ embryos exhibited an expression pattern of *STAR* that was more similar to *in vivo* tubular embryos (Fig. 3*a*). Expression levels of *CYP11A1* mRNA were increased ($P < 0.05$) in all *in vitro* treatment groups (CONT, ENC− and ENC+) compared with their initial *in vivo* spherical controls (Fig. 3*b*). Furthermore, *CYP11A1* expression was greater ($P < 0.05$) in ENC+ compared with CONT and ENC− embryos. Again, as observed with *STAR*, the expression pattern of *CYP11A1* in CONT and ENC− embryos was more similar to that in *in vivo* ovoid embryos, whereas ENC+ embryos exhibited an expression pattern of *CYP11A1* that was more similar to *in vivo* tubular embryos (Fig. 3*b*). Expression of *CYP19A1* mRNA was greater ($P < 0.05$) in ENC+ than CONT and ENC− embryos (Fig. 3*c*). In addition, *CYP19A1* expression was significantly greater ($P < 0.05$) in ENC+ embryos than *in vivo* spherical controls, whereas *CYP19A1* expression did not differ between CONT, ENC− and *in vivo* spherical control embryos (Fig. 3*c*). Furthermore, *CYP19A1* expression was decreased ($P < 0.05$) in

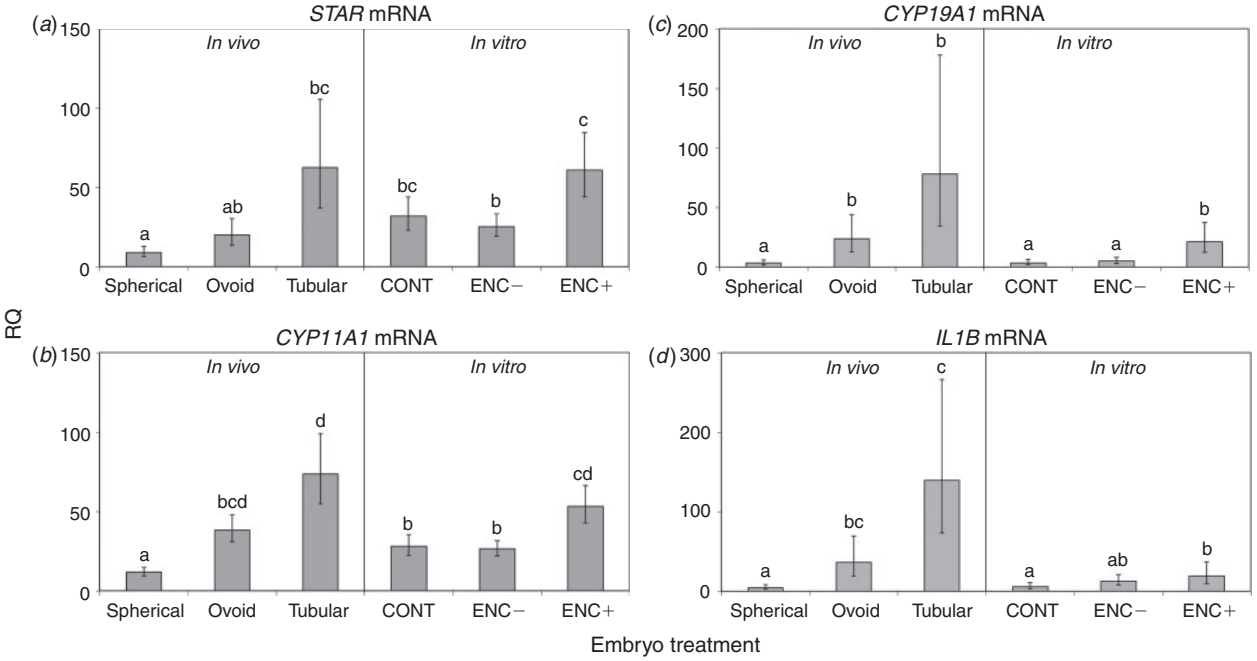

Fig. 3. Expression of (*a*) steroidogenic acute regulatory protein (*STAR*), (*b*) cytochrome P450 side chain cleavage (*CYP11A1*), (*c*) aromatase (*CYP19A1*) and (*d*) interleukin-1β (*IL1B*) mRNA, as determined by quantitative polymerase chain reaction, from *in vivo*-produced control embryos, *in vitro*-cultured non-encapsulated control embryos (CONT), encapsulated embryos with no observable morphological changes (ENC−) and encapsulated embryos with observable morphological changes (ENC+). Data were log transformed before analysis and then back transformed to observable values. Data show relative quality (RQ) expressed as the least-squares mean ± s.e.m. Statistical analysis demonstrated that embryo treatment had significant effects on the expression level of (*a*) *STAR* ($P < 0.01$), (*b*) *CYP11A1* ($P < 0.001$), (*c*) *CYP19A1* ($P < 0.01$) and (*d*) *IL1B* ($P < 0.01$). For each transcript, RQ values among treatment groups with different letters differ significantly ($P < 0.05$).

CONT and ENC− embryos compared with *in vivo* ovoid and tubular embryos (Fig. 3*c*). In contrast, *CYP19A1* expression did not differ between ENC+ and the later-stage *in vivo* ovoid and tubular embryos, although the expression pattern was numerically more similar in ENC+ and *in vivo* ovoid embryos (Fig. 3*c*). No significant differences were detected in the expression of *IL1B* mRNA between CONT, ENC− and *in vivo* spherical control embryos (Fig. 3*d*). However, *IL1B* expression was increased in ENC+ compared with CONT and *in vivo* spherical control embryos (Fig. 3*d*). There was no significant difference in *IL1B* expression between ENC+ and *in vivo* ovoid embryos, but *IL1B* expression was significantly ($P < 0.05$) decreased in ENC+ compared with *in vivo* tubular embryos.

Analysis of E2 and protein content

A significant time-dependent increase ($P < 0.001$) in E2 levels in the culture media of encapsulated embryos (both ENC+ and ENC−) was identified compared with culture media from CONT embryos and culture medium alone (Fig. 4*a*). Culture media from all embryo groups (i.e. CONT, ENC− and ENC+) had greater ($P < 0.05$) E2 levels than medium alone after 96 h of culture (Fig. 4*a*). Samples of culture medium from ENC+ embryos had significantly greater levels ($P = 0.05$) of E2 than samples from the CONT and ENC− groups after 72 and 96 h culture (Fig. 4*a*). In contrast, samples of culture medium from the ENC− group contained intermediate (but significantly different; $P < 0.05$) levels of E2 between ENC+ and CONT embryos after 96 h culture (Fig. 4*a*). A similar pattern of increased ($P < 0.05$) E2 production was observed in uterine flushings as pregnancy progressed from Day 9 to Days 10 and 11 of gestation, with the greatest E2 levels in uterine flushings on Day 11 of gestation (Fig. 4*b*).

There were no significant differences in total protein levels in samples of culture medium from the MEDIA, CONT, ENC− or

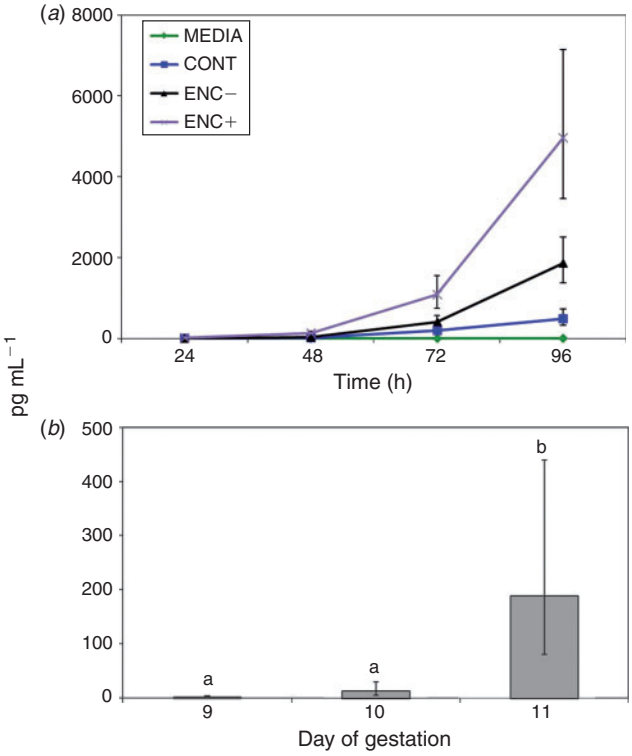

Fig. 4. (*a*) 17β-Oestradiol (E2) concentrations in samples of the culture medium collected at 24, 48, 72 and 96 h culture from wells containing medium only (MEDIA), non-encapsulated control embryos (CONT), encapsulated embryos with no observable morphological change (ENC−) or encapsulated embryos with observable morphological changes (ENC+). (*b*) E2 concentrations in uterine flushings from pregnant gilts on Days 9, 10 and 11 of gestation. Data were log transformed before analysis and then back transformed to observed values and are presented as the least-squares mean ± s.e.m. Statistical analysis demonstrated a significant culture media treatment × culture time interaction ($P < 0.001$) for E2 concentration in which samples of culture medium from ENC+ embryos had greater E2 concentrations than samples from the MEDIA, CONT and ENC− groups at 72 and 96 h. Statistical analysis demonstrated a significant gestational day effect ($P < 0.05$) for E2 concentrations in uterine flushings.

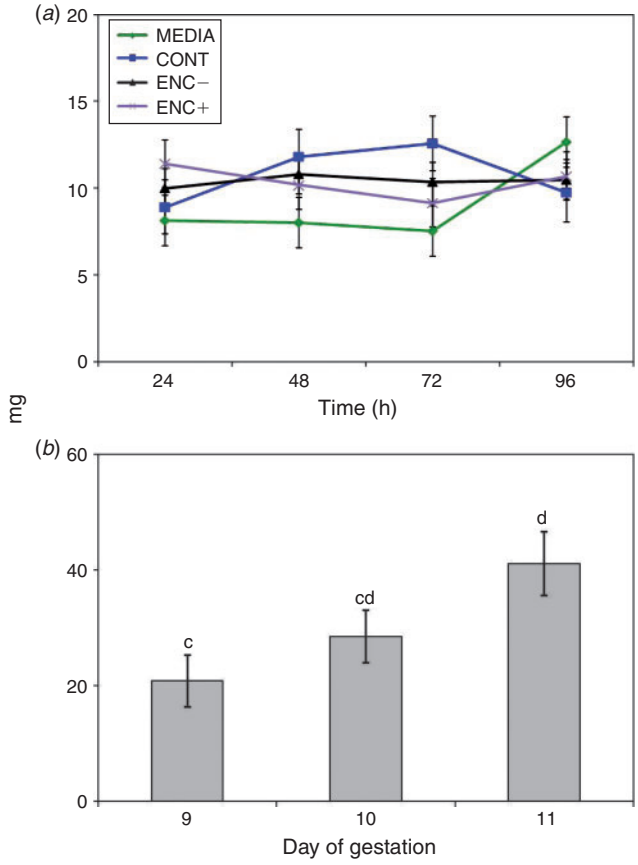

Fig. 5. (*a*) Total protein in samples of the culture medium collected at 24, 48, 72 and 96 h culture from wells containing medium only (MEDIA), non-encapsulated control embryos (CONT), encapsulated embryos with no observable morphological change (ENC−) and encapsulated embryos with observable morphological changes (ENC+). (*b*) Total protein in uterine flushings from pregnant gilts on Days 9, 10 and 11 of gestation. Data are presented as the least-squares mean ± s.e.m. Statistical analysis demonstrated no significant ($P > 0.10$) effects for total protein collected from the culture media at 24, 48, 72 and 96 h culture. Statistical analysis demonstrated a tendency ($P = 0.09$) for increased total protein in uterine flushings on Day 11 of gestation compared with Day 9, with Day 10 demonstrating an intermediate level of protein.

ENC+ groups (Fig. 5*a*). In contrast, there tended ($P = 0.09$) to be a time-dependent increase in total protein levels from uterine flushings as pregnancy progressed from Day 9 to Day 11 of gestation (Fig. 5*b*).

Discussion

In the present study, alginate hydrogels were used as 3D matrices to support *in vitro* culture of porcine embryos during the preimplantation period. Previously, alginate hydrogels have been used to support *in vitro* development of early blastocysts in cattle (Yániz *et al.* 2002), as an artificial zona pellucida for mouse morulae (Krentz *et al.* 1993) and for the culture of ovarian follicles in mice (Kreeger *et al.* 2006; Xu *et al.* 2006*a*, 2006*b*; West *et al.* 2007) and non-human primates (Xu *et al.* 2009). However, the present study is the first to demonstrate the use of alginate hydrogels for porcine embryo culture. For those studies involving the encapsulation of early bovine embryos, 1.5% alginate hydrogels supported similar blastocyst rates as 2D controls; however, the alginate system resulted in decreased blastocyst hatching rates, suggesting that the percentage of alginate may have formed a matrix that was too rigid to allow for hatching (Yániz *et al.* 2002). Studies involving mouse ovarian follicle culture investigated mechanical properties of alginate gels of varying concentrations and demonstrated that decreasing alginate matrix stiffness enhanced follicle growth (West *et al.* 2007), linking biomechanical environmental factors with cell and/or tissue development and function. Specifically, these studies demonstrated that 0.7% alginate, the lowest concentration of alginate tested, formed a low-stiffness hydrogel that was the most permissive to mouse follicle growth, cellular differentiation and corresponding steroid production (West *et al.* 2007). Based on these findings, 0.7% alginate was used in the present study to provide sufficient mechanical support to properly maintain porcine embryo development, without inhibiting growth or survival.

Our *in vitro* culture system using alginate hydrogels was able to facilitate morphological changes of preimplantation porcine embryos by applying a tissue engineering approach to embryo culture. Although survival rates did not differ between the encapsulated and non-encapsulated embryos, morphological changes were only observed with embryos encapsulated in alginate hydrogels. The inability of the non-encapsulated control embryos to undergo morphological changes *in vitro* suggests that a 3D matrix is required to maintain appropriate embryo architecture for proper development during the elongation stage of gestation. These results support the findings of previous studies that demonstrated that both mechanical forces, generated from the stiffness of surrounding tissues or substrates, and cell-generated contractility regulate differentiation during embryonic development (Wozniak and Chen 2009). Our *in vitro* culture system using alginate hydrogel as a 3D matrix may be initiating embryo morphological change by stimulating biochemical signals within the embryo via mechanotransduction (Vogel and Sheetz 2006; Wozniak and Chen 2009) or by simply providing support for the 3D structure of the embryo to maintain its cell-to-cell communication, a feature that traditional culture systems have been lacking. The requirement of the 3D structure as a necessity to support morphological changes can be supported further by the observation that when ENC+ embryos within our culture system exited the alginate hydrogel they formed a secondary spherical structure, likely due to fluid transport into the trophectoderm, which was observed in the non-encapsulated CONT embryos that did not have 3D support.

Although the percentage of total encapsulated embryos that exhibited morphological changes (17%) may seem lower than ultimately desired, it is important to emphasise that if the encapsulated embryo survives within our newly developed *in vitro* system, it has a relatively high chance of initiating morphological changes (32%) in simple basal medium conditions. In a previous study involving *in vitro* culture of bovine embryos in agarose gel tunnels, high levels of glucose in the culture medium may have resulted in rapid cell growth, contributing to the appearance of embryonic elongation (Brandão *et al.* 2004). Conversely, in the present study, no particular growth factors or nutrients were added to the culture medium, indicating that limited embryonic morphological changes can occur *in vitro* within alginate hydrogels without the addition of special media supplements. Although the percentage of porcine embryos initiating morphological changes in the present study is significantly less than reported in studies culturing bovine embryos in the agarose gel tunnel system, which ranged from 54% to 88% (Brandão *et al.* 2004; Vajta *et al.* 2004; Machado *et al.* 2012), it is important to highlight that these bovine studies with a high percentage of morphological changes were performed using embryos that were strictly produced *in vitro* and these embryos were selectively chosen as high-quality *in vitro*-produced embryos before being placed into the agarose gel tunnel system (Brandão *et al.* 2004; Vajta *et al.* 2004; Machado *et al.* 2012). In contrast, the present study only used *in vivo*-produced embryos collected on Day 9 of gestation in the pig. A previous study that investigated morphological changes using *in vivo*-produced bovine embryos within the agarose gel system reported a very low percentage (3%) of *in vivo*-produced bovine embryos that initiated morphological changes following *in vitro* culture in the agarose gel system (Machado *et al.* 2013). This finding, in conjunction with the findings of the present study, illustrates that *in vivo*-produced embryos are not as efficient as *in vitro*-produced embryos at initiating morphological changes within *in vitro* culture systems, which is likely the result of the dramatic environmental change that *in vivo*-produced embryos experience going from the uterine environment to an *in vitro* environment. Therefore, the percentage of embryos that underwent morphological changes in the present study (i.e. 17%) is relatively high for a study involving *in vivo*-produced embryos within an *in vitro* culture system, with only basal culture medium.

Several transcripts, such as steroidogenic transcripts (i.e. *STAR*, *CYPY11A1* and *CYP19A1*) and immune response transcripts (i.e. *IL1B*), are increased in a similar pattern as oestrogen production during embryo elongation (Yelich *et al.* 1997; Blomberg *et al.* 2005). According to the results of the present study, *STAR* expression levels in ENC+ embryos were similar to those in tubular *in vivo* embryos, but greater than in ovoid *in vivo* embryos. In contrast *STAR* expression in CONT and ENC− embryos remained similar to that in ovoid *in vivo* embryos. *STAR*, which is a major initiator of steroidogenesis

(Stocco and Clark 1997), has previously been found to be more abundantly expressed in elongated filamentous embryos than in ovoid embryos (Blomberg *et al.* 2005). Therefore, the *STAR* expression results of the present study suggest that embryos undergoing morphological changes within alginate hydrogels exhibit similar *STAR* expression trends as those that occur during initiation of elongation *in vivo*. Similar gene expression patterns were found for *CYP11A1* and *CYP19A1* within our *in vitro* culture system; these two are the additional transcripts encoding for rate-limiting proteins required for oestrogen synthesis (Blomberg *et al.* 2005). Expression of *CYP11A1* and *CYP19A1* was increased in ENC+ compared with CONT and ENC− embryos. These findings further suggest that encapsulated embryos that transformed morphologically during culture also changed at the level of gene expression, corresponding to the morphological transition and differential steroidogenic gene expression that occurs during the onset of elongation *in vivo*.

Proper interactions between the embryo and receptive uterine endometrium are also essential for supporting embryonic development and subsequent implantation (Ross *et al.* 2003). These interactions are initiated by the immune response cytokine *IL1B*, which is increased by the embryo during elongation and may be responsible for preventing conceptus rejection by suppressing the maternal immune response (Ross *et al.* 2003). Although our results demonstrate that the expression of *IL1B* was greater in ENC+ compared with CONT embryos, the expression of *IL1B* was decreased in ENC+ compared with tubular *in vivo* embryos. This outcome may suggest that direct interaction with the maternal endometrium is likely required for upregulation of *IL1B* in the embryo, whereas upregulation of steroidogenic transcripts may be independent of maternal–embryonic cross-talk.

In vivo oestrogen production by the porcine embryo increases during the elongation stage, stimulating the synthesis and release of numerous endometrial secretory proteins, which is essential for establishing maternal recognition of pregnancy (Geisert *et al.* 1982*b*; Yelich *et al.* 1997). Our results indicate that the ENC+ embryos produce and secrete a greater amount of E2 at 72 and 96 h of culture compared with the CONT and ENC− embryos. In addition, this upregulation of E2 in the culture medium of ENC+ embryos was independent of increased production of total protein within the culture medium. These results correspond with the increased expression of steroidogenic transcripts *STAR*, *CYP11A1* and *CYP19A1* in the ENC+ embryos, which supports previous findings of the direct correlation between these transcripts and steroid synthesis (Stocco and Clark 1997; Blomberg *et al.* 2005). Therefore, the E2 results further suggest that embryonic morphological changes induced by the alginate hydrogel culture system are indications of the initiation of elongation due to the corresponding increase in oestrogen production. The time-dependent increase in E2 observed in culture media of ENC+ embryos followed a similar trend as observed for E2 levels in the uterine flushings as pregnancy advanced through this time period, with the greatest production of oestrogen observed on Day 11 of gestation when tubular embryos were present. Furthermore, protein increased in the uterine flushings as pregnancy advanced. Given that protein increases within the uterine milieu regardless of pregnancy status (Vallet *et al.* 1996), increased expression of steroidogenic transcripts and E2 production by ENC+ embryos without a difference in protein levels within the culture medium further suggests that upregulation of steroidogenesis and subsequent oestrogen production may be independent of maternal–embryonic cross-talk. It is important to highlight that there was a 48-h delay for embryos cultured within alginate hydrogels to initiate production and secretion of E2 into the culture medium, as well as for significant morphological changes to occur, which indicates a delay in development for *in vitro*-cultured compared with *in vivo*-developed embryos. A similar delay in morphological changes was observed in bovine embryos cultured in the agarose gel system compared with *in vivo*-developed embryos (Machado *et al.* 2013). This delay is likely due to the embryos recovering from the encapsulation technique and adjusting to the *in vitro* microenvironment.

Unlike elongation in other domestic animals, rapid elongation of the pig embryo has been primarily associated with cellular remodelling and differentiation rather than hyperplasia (Geisert and Yelich 1997). As a result, follow-up studies investigating potential markers for differentiation of the trophectoderm and mesoderm would be useful for measuring the level of differentiation from embryos undergoing morphological changes within our *in vitro* culture system. Furthermore, embryonic disc establishment, which was not monitored in the present study, will be included in follow-up studies to further evaluate the efficiency of our culture system. Monitoring the formation of the embryonic disc will be a useful technique for measuring the synchrony between trophoblast and embryonic disc development as we move forward to improve our *in vitro* culture system using alginate hydrogels.

In conclusion, the present study demonstrates an *in vitro* culture system that can support porcine embryo development during initiation of elongation. Our culture system, which uses alginate hydrogel as a 3D extracellular matrix for porcine embryo culture, has been shown to facilitate embryonic morphological changes with corresponding increases in steroidogenic transcript expression and oestrogen production, in a similar pattern as observed in *in vivo*-developed embryos. Our findings advocate the potential of our alginate culture system as an *in vitro* tool for evaluating and understanding specific mechanisms of embryo elongation. Further investigations with our 3D *in vitro* culture system can focus on the effects of certain growth factors, hormones, ligands and uterine epithelial cell coculture on the development of preimplantation porcine embryos. Specific factors regulating pig embryo elongation can be identified to develop strategies to improve pregnancy outcomes in the pig. Factors identified could then be manipulated through either direct intervention or marker-assisted selection of genomic markers to improve early embryonic survival, uterine capacity and preweaning piglet survival, which could have a significant impact on the profitability of swine production. Furthermore, our alginate culture system could be applied to embryos of other ungulate species, such as cattle and sheep, that also undergo embryo elongation during the preimplantation period of pregnancy to identify potential regulators of normal embryonic development in a variety of species.

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